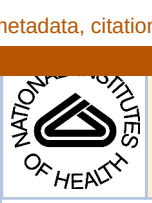

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Tumor Suppressor Mechanisms in Immune Ageing

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SUMMARY

The cancer-ageing hypothesis suggests that the activation of some tumor suppressor mechanisms beneficially prevent cancer but also untowardly promote mammalian ageing. Along these lines, activation of tumor suppressor mechanisms that inhibit the cell cycle (*e.g.* $p16^{INK4a}$ and p53) in response to DNA damage and other age-promoting stimuli have taken center stage in immune-ageing research. Immune cells are intrinsically susceptible to transforming events due to V(D)J recombination, a high rate of cellular turnover and requisite long-term self-renewal. Therefore, the DNA damage response and cell cycle regulation play a clear role in maintaining homeostasis without neoplastic progression. Here we will argue based on recent advances in our understanding of tumor suppressor mechanisms in immune cells, however, that aspects of these same beneficial pathways have the potential to induce intrinsic immune ageing.

INTRODUCTION

Aspects of mammalian ageing appear to result from the persistent activation of related tumor suppressor mechanisms by diverse gerontogenic stimuli. Such age-promoting signals probably include environmental mutagens (e.g. UV radiation), as well as intrinsic replicative and metabolic stresses (e.g. reactive oxygen species (ROS)). DNA damage, either alone, or downstream of some of these stresses is associated both with ageing and the activation of tumor suppressor mechanisms (Figure 1); however, the exact stimuli that promote their activation in ageing are unknown.

Immune cells possess unique characteristics that make them susceptible to malignant transformation. Both T and B lymphocytes undergo dramatic proliferation and V(D)J recombination to obtain their massive antigen receptor diversity during their development. Upon pathogen encounter, they engage in clonal expansion and B lymphocytes also go through somatic hypermutation to achieve higher BCR affinity. Additionally, memory lymphocytes undergo self-renewal, and maintain impressive replicative capacity in response to antigenic challenges, even after decades without such stimulation. As a result, the DNA damage response and other tumor suppressor mechanisms appear to be finely regulated to allow for this unusual proliferative behavior.

Here, we will argue that activation of tumor suppressor pathways in response to age-promoting ("gerontogenic") stimuli promote immune ageing by inducing a proliferative compromise, perhaps even cellular senescence, or an increased propensity to undergo apoptosis; and we

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suggest these cell-intrinsic alterations contribute to ageing of the immune system. It is also important to note that cell extrinsic factors, such as degradation of immunologic "niches" and alterations of the hormonal milieu undoubtedly play an important role in mammalian immune ageing. For example, thymic involution contributes to reduced thymic output with ageing (reviewed in [1]) and hematopoietic stem cell (HSC) ageing is partially determined by the bone marrow microenvironment [2]. Although such extrinsic mechanisms are of doubtless physiologic importance in ageing, in this review, we will focus on cell-intrinsic ageing of the immune system that results from tumor suppressor activation.

The induction of age-promoting tumor suppressor mechanisms

A common feature of several "gerontogens" is the induction of DNA damage with subsequent initiation of DNA Damage Response (DDR) pathways. Alteration in DNA metabolism is associated with features of premature ageing in a few human genetic diseases (*e.g.* Werner syndrome [3]), as well as several mouse models (reviewed in [4]). These data have advanced the hypothesis that DNA damage and/or the cellular responses activated as part of the DDR promote ageing. One of the most important responders of the DDR is the p53 tumor suppressor, which is activated by a series of kinases that sense several forms of DNA damage (Figure 1). Additionally, p53 can also be stabilized by "mitogenic stress" through induction of the Arf tumor suppressor gene, and this is thought to occur largely independent of DNA damage [5]. Arf is transcribed in an 'Alternate Reading Frame' with another tumor suppressor gene, $p16^{INK4a}$, that is also thought to regulate ageing (reviewed in [6]). Importantly, the p53 transcription factor engages a diverse set of cellular pathways which can lead to a variety of outcomes including enhanced DNA repair, a transient cell cycle arrest, a permanent growth arrest (*i.e.* senescence), or apoptosis depending on the quality, intensity and persistence of the damaging signal (Figure 2). Given these diverse possible outcomes of p53 expression, it is perhaps not surprising that p53 has been suggested to both accelerate and retard mammalian ageing when activated in different contexts in murine systems [7–10••]

The induction of the $p16^{INK4a}$ tumor suppressor gene is less well-understood. Several diverse stresses have been shown to increase $p16^{INK4a}$ expression, among them oxidative stress, oncogene activation and telomere dysfunction (Figure 1). Expression of $p16^{INK4a}$ leads to decreased activity of the CDK4/6 proliferative kinases with attendant RB hypophosphorylation and growth arrest in some, but certainly not all, proliferating cell types [11,12]. Under some circumstances, $p16^{INK4a}$ activation and persistent RB hypophosphorylation can induce cellular senescence [6]. It is worth noting that by "cellular senescence", we refer to a specialized, permanent form of growth arrest originally described by Hayflick and colleagues. This should not be confused with "immunosenescence" and related terms used generically to describe cellular hypoproliferation noted in the ageing immune system. Cellular ('Hayflickian') senescence represents a precise cell biological phenotype where cells are irreversibly arrested in G1 phase, but remain metabolically active, and viable indefinitely [13]. In contrast, "replicative senescence" of lymphocytes has been used to refer to limited proliferation upon mitogen or antigen stimulation seen in cells from aged organisms [14]. Importantly, expression of $p16^{INK4a}$ has been closely linked to the Hayflickian senescence of cultured human cells, whereas the importance of $p16^{INK4a}$ for "immunosenescence" is an area of ongoing investigation. For the purposes of clarity, in this review, we will reserve the term "senescence" for the more precisely defined, permanent growth arrest described by Hayflick.

Does telomere dysfunction cause immune ageing?

Telomere dysfunction appears to increase in primates with physiological ageing [15], and has been linked to p53 and $p16^{INK4a}$ activation. Telomeres are nucleoprotein structures that act to protect chromosome ends from degradation. They are composed of numerous 5′ TTAGGG 3′ repeats (in vertebrates), a 3′ G-rich overhang and the protein complex named shelterin

(reviewed in [16]). Telomeres shorten with each round of cell division due to the inability of DNA polymerase to completely copy terminal linear DNA sequences. Telomere shortening is thought to limit proliferative capacity, and therefore may serve as a barrier to turmorigenesis, at least in the context of intact p53 function. Telomerase is a specialized RNA-protein complex that synthesizes telomere repeats *de novo* and maintains telomere length during cell division. Telomerase is expressed in germ cells but is absent in most human somatic tissues [5]. Critically shortened telomeres cannot be bound by the shelterin complex and form uncapped telomere ends, which are subsequently recognized as double strand breaks (DSBs) and cause initiation of DDR.

Several lines of evidence have suggested that telomere dysfunction may contribute to human ageing. Telomerase-deficient mice that have been serially backcrossed to harbor human-length telomeres demonstrate regenerative failure in multiple organs due to a decline in stem cell proliferative capacity and tissue repair ability [17], showing that telomere dysfunction can induce aspects of the impaired tissue regeneration characteristic of human ageing. Likewise, while humans with WRN deficieny exhibit a profound progeria, Wrn-deficient mice do not appear progeroid except in the setting of shortened telomeres [18]. In the immune system, telomerase is required to maintain HSC lifespan during serial transplantation [19] and to prevent ageing-related abnormal B cell and myeloid hematopoiesis in mice [20,21••]. In addition, human CD28 negative T cells, which have lost optimal telomerase activity, accumulate *in vivo* with age and display impaired function [22], and the overexpression of telomerase or activation of telomerase using small molecules partially rescues these defects [22,23•]. Patients with dyskeratosis congenita (DKC) and other diseases that harbor genetic defects in telomerase activity also display some features of premature ageing (reviewed in [16]). In particular, some DKC patients exhibit immune ageing phenotypes, such as lymphopenia and lymphocyte hypo-proliferation [24].

On the other hand, evidence has suggested that many aspects of ageing can occur in the absence of telomere dysfunction, and it is not clear if telomeric signals usually contribute to human ageing under physiological settings. For example, mean telomere length correlates poorly with ageing [25,26], and telomere shortening is only weakly associated with most common human age-associated diseases [27–29]. Secondly, while normal inbred mice demonstrate many familiar aspects of mammalian ageing, this appears to be almost entirely telomere-independent, as telomerase-deficient mice demonstrate only subtle abnormalities with regard to ageing [17]. Moreover, as in Terc-deficient mice, phenotypes of telomerase deficiency in humans are generally seen in patients who also inherit shortened telomeres from parents with telomerase deficiency (termed ‘anticipation’) (reviewed in [16]). Thirdly, other than perhaps cirrhosis, classic ageing phenotypes such as atherosclerosis, type 2 diabetes mellitus, osteopenia, or neurodegenerative disease have not yet been reported in human patients with telomerase deficiency. That is, humans harboring sufficient telomere dysfunction to induce overt disease such as aplastic anemia and pulmonary fibrosis do not exhibit a segmental progeria akin to that seen in WRN patients. We believe these data for and against the role of telomere dysfunction in human ageing, when taken in aggregate, suggest that telomere dysfunction likely contributes to age-associated phenotypes in humans, but it still remains unclear as to whether telomere dysfunction plays a major or minor role in these processes.

Complex roles in ageing for the p53 pathway

As mentioned, activation of p53 can induce a variety of responses including transient cell cycle arrest (often accompanied with DNA repair), senescence or apoptosis. Correspondingly, recent studies in mouse models have indicated that the effect of p53 activation on ageing is complex (Figure 2). In some murine models, activation of p53 appears to generally accelerate ageing [7,8]. With regard to immune function, deletion of p53 in mice increases the number and

abrogates the age-related decline of HSC self-renewal capacity [30–32]. Likewise, increased p53 dosage causes reduced repopulating capacity in HSCs [10••]. Deletion of p53 rescues the ageing phenotype of stem cells in mice with dysfunctional telomeres, but fails to extend the life span of these animals due to increased tumor susceptibility [33]. In contrast, inactivation of $p21^{CIP}$, a p53-target that induces cell cycle arrest, rescues proliferative defects of stem cells in telomerase deficient mice and extends their longevity [34]. These data suggest a pro-ageing role of the p53 pathway in certain settings.

In other systems, however, p53 activation has been shown to delay ageing. Mice harboring extra copies of the *p53* and *Ink4a/Arf* genes (super *Ink4a/Arf*/*p53* mice) display an extended longevity, decreased malignancy and enhanced protection from DNA damage [9••,35], while mice have reduced gene dosage of MDM2 or an extra copy of either ARF or p53 do not exhibit accelerated ageing (reviewed in [35]). Consistent with these data, inactivation of Arf in a premature ageing mouse model (BubR1 insufficient) leads to exacerbated ageing phenotype, while loss of $p16^{INK4a}$ rescues aspects of this progeria [36•]. Also of interest, p53-deficient mice show accelerated ageing in the T cell compartment [37]. These data suggest Arf-p53 function can be anti-ageing, presumably by augmenting DNA repair and therefore preserving genomic integrity.

These two seemingly contradicting roles of p53 in ageing may result from differences in the quality or persistence of the stimuli that activate p53 expression. Some activating signals may skew its response towards transient arrest and DNA repair, thereby restoring normal homeostasis and acting as an anti-ageing checkpoint. Additionally, the persistence of the stimuli that induces p53 may be important. Long-term p53 activation, as occurs with telomere dysfunction, might promote senescence or cell death, whereas transient activation might enhance genomic fidelity. In accord with these views, the progeroid p53 overexpresing mice [8,10••] appear to have a greater and more persistent degree of p53 activation compared to the super-p53 mice [9••,35] which do not show accelerated ageing. Correspondingly, loss of the p53 target gene, $p21^{CIP}$, which induces an important, transient cell cycle pause after DNA damage compromises long-term HSC function during serial transplantation [38–40••], but significantly enhances HSC function in the setting of persistent telomere dysfunction [34]. We believe the murine data suggests that the effects of the pleiotropic p53 transcription factor with regard to ageing depend on the nature and duration of the stresses that engage p53 in vivo.

$p16^{INK4a}$-pRB pathway and senescence

The $p16^{INK4a}$ tumor suppressor is an important mediator of cellular senescence, and recent work has suggested it may also play a role in human ageing [6] (Figure 3). Expression of $p16^{INK4a}$ is induced upon T cell activation and has been shown to possibly cause hypo-proliferation before cells reach permanent, possibly senescent growth arrest *in vitro*, in which T cells cease responding to stimulation in culture [41]. These observations raise the critical question as to whether $p16^{INK4a}$ can promote ageing by inducing hypoproliferation (e.g. in T cells) without necessarily causing true ‘Hayflickian’ senescence *in vivo*.

The expression of $p16^{INK4a}$ has been shown to sharply increase with ageing in the majority of human, baboon and rodent tissues tested to date [17]; and caloric restriction, which retards ageing in rodents, attenuates age-induced increase in $p16^{INK4a}$ expression [42,43]. Recent studies [44–47••] have demonstrated that increased $p16^{INK4a}$ expression in mice causes an age-associated functional decline in HSCs, pancreatic islet cells, neural stem cells (NSC), and B lymphocyte progenitors [47••]. Accordingly, $p16^{INK4a}$ deletion attenuates the age-related decline in the repopulation potential of stem cells in these compartments [44–46] and partially rescues premature ageing in skeletal muscle and fat tissues in a premature ageing mouse model (BubR1 insufficient) [36•].

Recent evidence has also linked $p16^{INK4a}$ expression to human ageing. For example, we have recently shown that $p16^{INK4a}$ expression in human peripheral blood T cells increases sharply with ageing and strongly correlates in independent samples with gerontogenic behaviors such as smoking and physical inactivity [48•]. Moreover, findings from human association studies have recently linked $p16^{INK4a}$ expression to several human age-related phenotypes. Single nucleotide polymorphisms (SNPs) whose genotypes are associated with the risk of age-related conditions, such as frailty, type 2 diabetes mellitus, stroke and myocardial infarction, have been mapped by candidate approaches and genome-wide studies to within 120 kb of the human *INK4/ARF* locus (reviewed in [17]). We have recently identified that the expression level of $p16^{INK4a}$ and other *INK4a/ARF* transcripts in peripheral T-cells is strongly correlated with a common, non-coding SNP ~100 kb away from p16 ^{INK4a} promoter [49]. Although the precise mechanistic linkage between these 9p21 SNPs and human ageing are still unclear, these data provocatively suggest an important link between $p16^{INK4a}$ expression and human ageing.

Precisely what aspect of mammalian ageing induces $p16^{INK4a}$ expression is unknown. However, $p16^{INK4a}$ activation has been shown to be regulated at transcriptional and posttranscriptional levels (Figure 3). MAPK signaling via ERK and p38MAPK has been suggested to induce $p16^{INK4a}$ expression in response to oncogenic activation or stress stimuli. Expression of $p16^{INK4a}$ can be either transcriptionally activated (e.g. Ets family) or inhibited (e.g. Id proteins) by a number of pathways (reviewed in [6]). Although the majority of research has focused on transcriptional control, recent studies have suggested that $p16^{INK4a}$ can be regulated post-transcriptionally through interaction with microRNAs [50]. A particular link has been suggested between $p16^{INK4a}$ expression and PcG function. The PcG proteins BMI-1 [51–53], EZH2 [54–56•] and CBX7 [57] have been shown to act as epigenetic repressors of $p16^{INK4a}$ expression. Consistent with these data, Bmi-1 deficient mice display accelerated HSC attrition [53,58], reduced self-renewal of neural stem cells (NSC) [52], postnatal growth retardation and neurological defects [59], and diminished β-cell mass and phenotypes of T2DM [60•]. Importantly, the defects in HSC and NSC observed in Bmi-1 deficient animals are partially reversed by deletion of *Ink4a/Arf* [51,52,61,62]. Recent data has further suggested that an age-induced loss of PcG function may contribute to the increased expression of $p16^{INK4a}$ seen with ageing. For example, loss of Ezh2 and Bmi-1 binding to the *INK4a/ARF* locus has been reported with prolonged fibroblast culture with attendant increased expression of the locus in senescent cells [55]. Most recently, loss of EZH2 expression has been reported in pancreatic β-cells *in vivo* with ageing in humans and mice [56•]. Furthermore, specific somatic deletion of Ezh2 in this tissue causes *Ink4a/Arf* overexpression, a relative failure of insulin secretion and hyperglycemia; which can be rescued by *Ink4a/Arf* deficiency. These results suggest the possibility that a loss of PcG repression with ageing contributes to the observed increase in expression of $p16^{INK4a}$ noted in several human tissues.

With regard to immune ageing, besides its role in limiting HSC and B cell progenitor proliferation potential with ageing, $p16^{INK4a}$ has been shown to play an important role in immune cell development [63] and myeloid cell senescence [64]. Overexpression of $p16^{INK4a}$ in T cells arrests thymocyte development at the DN stage presumably through induction of hypo-proliferation during thymocyte differentiation [63]. Consistent with this result, $p16^{INK4a}$ mediates peripheral T cell replicative senescence/hypo-proliferation [41,65] and $p16^{INK4a}$ deficient mice have increased thymocyte and peripheral T cell number [66,67]. However, it is not known whether $p16^{INK4a}$ plays an intrinsic role in the ageing-related functional declines observed in terminally differentiated lymphocytes. It is recognized that immunological microenvironments play an essential role in the age-related changes documented for lymphocyte development and function [1]. However, current mouse models do not allow us to determine whether $p16^{INK4a}$ functions intrinsically or extrinsically in naive and memory lymphocyte ageing. Long-term bone marrow transplantation experiments or

future mouse models with lineage-specific deletion of $p16^{INK4a}$ are needed to address these questions.

CONCLUSIONS

In summary, the activation of tumor suppressor mechanisms in immune cells appears to provide an important defense against malignant transformation caused by DNA damages and other stimuli during their development, homeostatic replication and activation. Activation of these tumor suppressor mechanisms can lead to both pro- and anti-ageing effects in immune cells. For example, transient p53 activation likely induces a beneficial cell cycle pause and opportunity for DNA repair that affords a resistance to certain age-promoting stimuli. In contrast, gerontogenic stresses such as telomere dysfunction and other forms of persistent DNA damage appear to activate apoptosis and senescence via p53 and $p16^{INK4a}$ induction, which appears to be pro-ageing in some settings.

Major challenges going forward will be to understand immune ageing on a tissue, rather than cellular, level: that is, how these cell-intrinsic activities influence the behavior of the affected cells and interact with cell-extrinsic changes to produce organismic immune ageing. For example, $p16^{INK4a}$ expression has been noted in several subsets of lymphocytes with ageing, but we think it is unlikely that all types of proliferation in these diverse subsets of lymphocytes are affected by $p16^{INK4a}$ expression. More likely, we think that certain lymphocyte proliferative events will be specifically retarded by an age-induced increase in $p16^{INK4a}$ expression, whereas the replicative capacity of other lymphocyte subsets may not be adversely compromised, or perhaps even increased, in the setting of $p16^{INK4a}$ induction. Likewise, an age-induced decline in the replicative capacity of certain cell types (e.g. T regulatory cells), may extrinsically lead to increased proliferation in other immune subsets. Sorting out the physiology of immune ageing will require carefully designed murine studies using conditional alleles, as well as more longitudinal studies of human immune ageing.

Another important issue is whether the pro- or anti-ageing effects of p53 predominate in humans. In this regard, it is important to note that conclusions about p53 regulation from mice may be of limited applicability to humans. There are important differences in ARF regulation and telomere biology between humans and rodents that may be of relevance to the role of p53 in ageing. As mentioned, telomere length is shorter and telomerase activity is more tightly regulated in humans compared to rodents, and therefore telomere dysfunction may be an important activator of p53 in human, but not rodent, ageing. Likewise, while Arf expression increases markedly with ageing in most rodent tissues [42,68] with a correlative increase in the expression of p53 target genes [42,43], expression of ARF has not been observed to increase with age in any human tissue tested to date, including T lymphocytes [48•] and kidney [69]. Likewise, human ARF does not increase with the onset of cellular senescence, nor is human ARF induced by ectopic RAS expression *in vitro*; whereas murine Arf markedly increases in expression during these settings (reviewed in [6]). It is tempting to speculate that species differences in the PcG repression of the *INK4a/ARF* locus [54,55] account for these differences in ARF regulation with ageing, although other explanations are also possible. Nonetheless, these major differences between mouse and man with regard to telomere and ARF biology suggest that care must be taken in drawing conclusions about the role of p53 in human ageing based on murine studies.

In summary, we believe the activation of tumor suppressor genes and cell cycle inhibitors influences immune ageing, and likely contributes to a cell-autonomous decline in cellular replicative capacity in specific immune cell subsets (e.g. B-cell progenitors [47••]). We believe future studies of immune ageing will allow for the identification of reliable molecular markers of molecular ageing to assess the progression of immune ageing and possibly forecast the onset

of certain age-associated diseases. Moreover, an enhanced understanding of the pro- and anti-ageing roles of tumor suppressor activation may provide therapeutic opportunity. Although we remain concerned that efforts to attenuate tumor suppressor responses risk enhanced neoplasia, we anticipate that a more sophisticated understanding of cellular ageing will permit the design of rational anti-ageing therapeutics to retard the pace of immune ageing.

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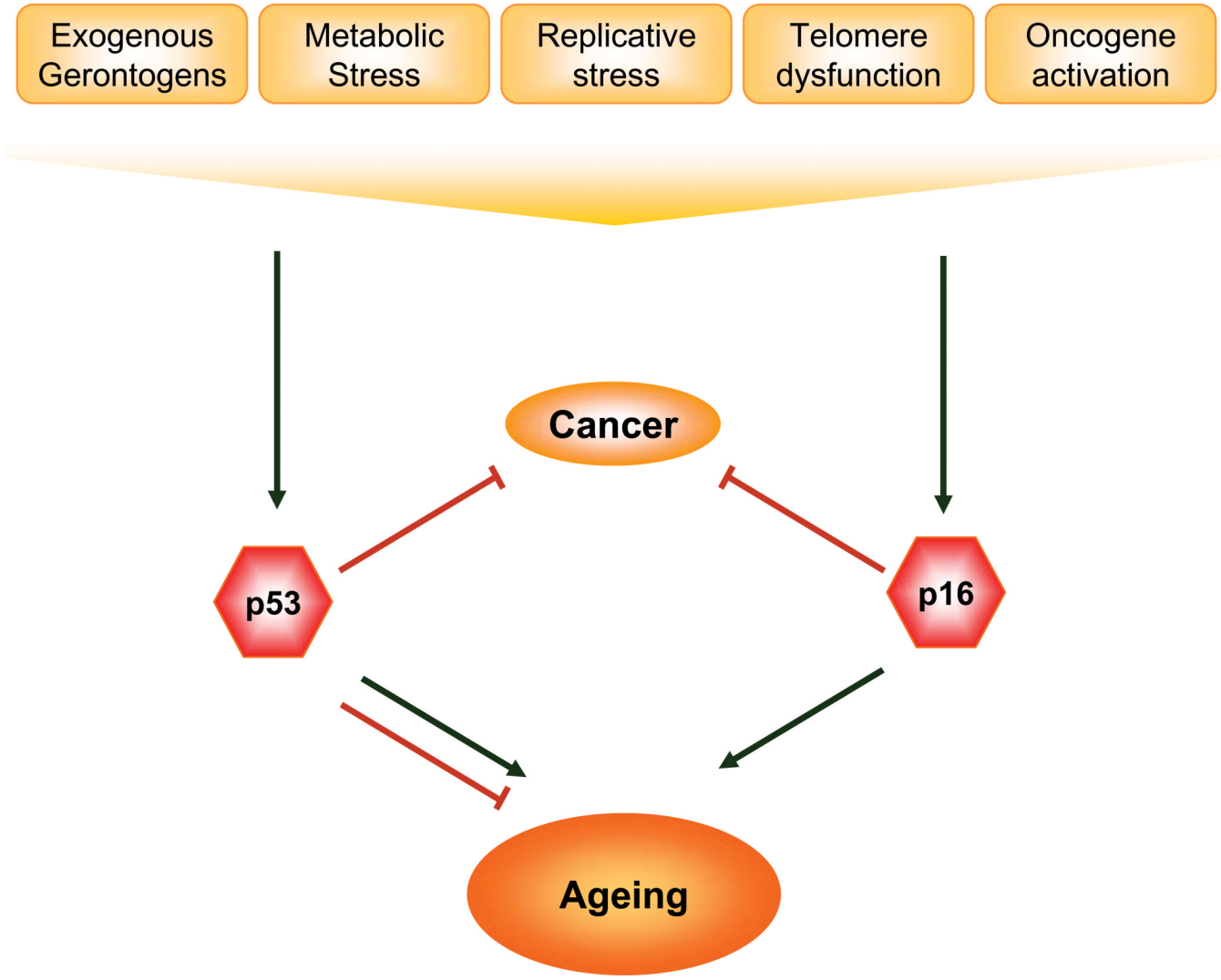

Figure 1. Tumor suppressor mechanisms and their role in cancer and ageing

In response to exogenous gerontogens and endogenous damage signals, the tumor suppressors p53 and $p16^{INK4a}$ are activated to prevent cancer but can also untowardly promote mammalian ageing through inducing cell growth arrest, senescence and apoptosis. Activation of p53, however, can also engage a transient cell cycle arrest and DNA repair that may retard ageing.

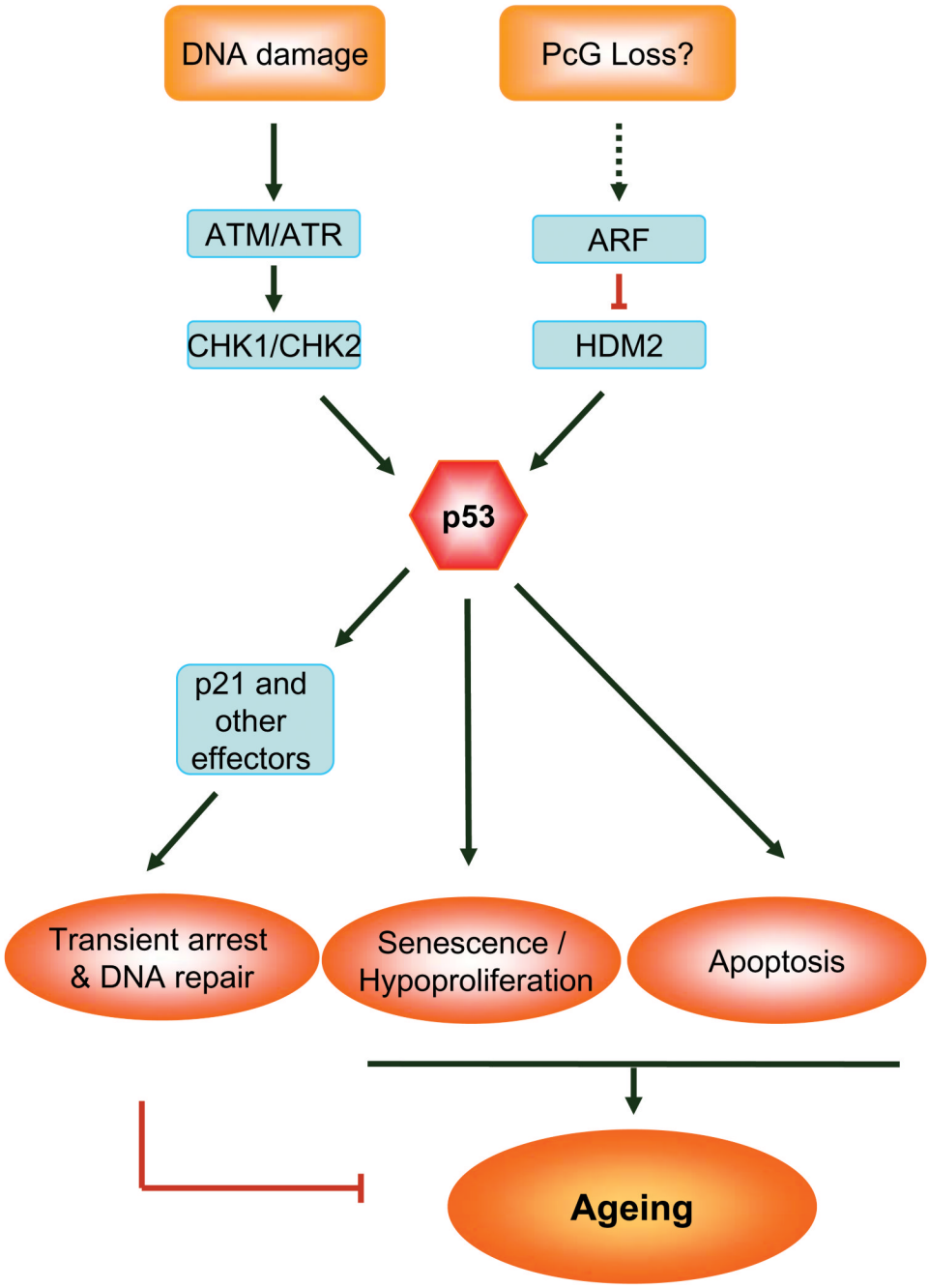

Figure 2. Activation of p53 pathway in immune ageing

Many age-promoting stimuli can lead to activation of p53 pathway through ATM/ATR-dependent DDR pathway or a DNA damage independent ARF-HDM2 pathway. ATM and/or ATR, together with CHK1 and/or CHK2, facilitate activation of p53 in response to DNA damage. In addition, p53 activation can also be induced by ARF in response to oncogene activation, PcG loss or other unknown mechanisms. ARF inhibits HDM2 (MDM2 in mice) and stabilizes p53 expression. Among a suite of p53 targets, $p21^{CIP}$ is activated and causes a transient cell cycle arrest. Apoptosis and senescence can also be triggered through other p53 downstream pathways. Both apoptosis and cellular senescence lead to ageing at the organism level, while transient arrest and DNA repair could restore normal homeostasis and delay ageing.

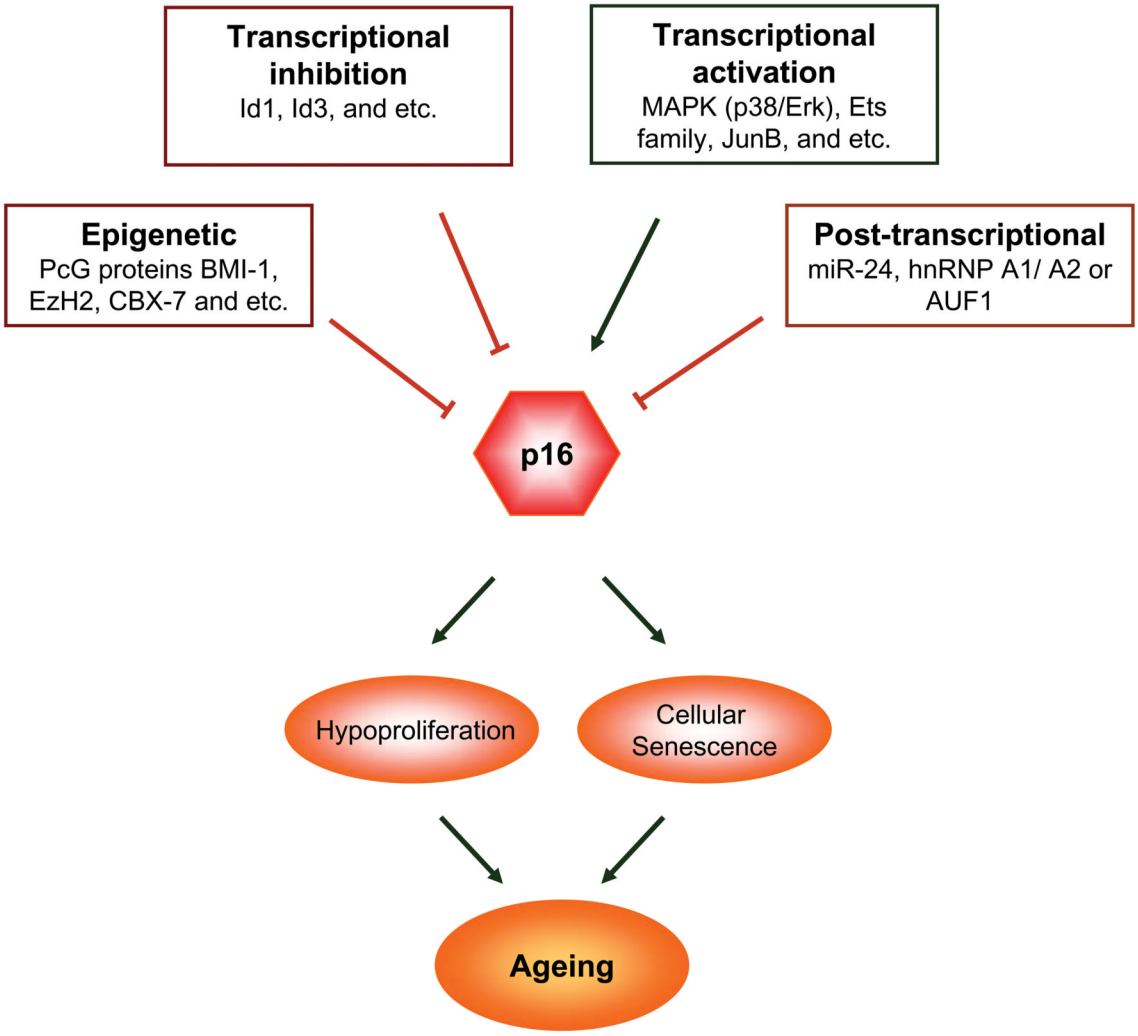

Figure 3. Regulation of p16^{INK4a} and its role in immune ageing

Senescence-inducing signals (e.g. mitogen stimuli and other unknown ageing-promoting stimuli) activate the p16^{INK4a} pathway by upregulating p16^{INK4a} expression, which can be achieved through a variety of mechanisms, including epigenetic, transcriptional, and posttranscriptional regulation. It is not clear whether different stress-related signals employ one or more specific mechanisms to regulate p16^{INK4a} expression. However, once activated, p16^{INK4a} inhibits the proliferative kinases CDK4/6, leading to immune ageing through induction of hypo-proliferation, and perhaps cellular senescence.